UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/516,983	12/03/2004	Luppo Edens	BJS-4662-357	1387

23117 7590 01/15/2008
NIXON & VANDERHYE, PC
901 NORTH GLEBE ROAD, 11TH FLOOR
ARLINGTON, VA 22203

EXAMINER	
LILLING, HERBERT J	
ART UNIT	**PAPER NUMBER**
1657	

MAIL DATE	DELIVERY MODE
01/15/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Interview Summary	Application No.	Applicant(s)	
	10/516,983	EDENS ET AL.	
	Examiner	Art Unit	
	HERBERT J. LILLING	1657	

All participants (applicant, applicant's representative, PTO personnel):

(1) *HERBERT J. LILLING*. (3)_____.

(2) *ATTORNEY B.J. SADOFF*. (4)_____.

Date of Interview: *08 January 2008*.

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☒ No.
If Yes, brief description: _____.

Claim(s) discussed: *NONE*.

Identification of prior art discussed: *Ito et al & Pfister et al*.

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☒ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *See attachment.*.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

ATTACHMENTS
1) Ito et al
2) Pfister et al

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

Examiner's signature, if required

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)
In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

ATTACHMENT TO INTERVIEW OF JANUARY 08, 2008

Attorney B.J. Sadoff requested information for this application which status is a Final Rejection to obtain the two prior art references which are not legible or incomplete after which time of receiving the prior art discuss the following issues in a future:

a) grounds for the rejection over the art of record;

b) possible amendments to the claimed subject matter which would overcome the art of record.

Both of the references, Ito et al and Pfister, after the interview were found to be available on the web in PDF form, one of which was emailed to Attorney , [Ito et al].

The issue with respect to the claimed product is that the prior art as alleged by this Examiner teaches compositions within the scope of having tripeptides containing terminal Pro-x-x as clearly indicated by the references.

Applicant may telephone this Examiner any time to discuss the application. Examiner will accept any arguments or amendments to the claims which would render the application allowable without any new unreasonable search for this after final rejection.

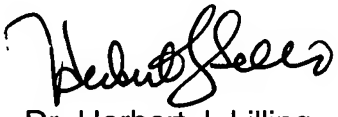
Applicant indicated that a response is due at the month of January.

Copies of the two references will be submitted with this interview summary which will be sufficient time for Attorney to obtain copies if Attorney is not able to obtain copies by the internet.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Examiner Lilling whose telephone number is 571-272-0918 and Fax Number is **571-273-8300**. or SPE Jon Weber whose telephone number is 571-272-0925. Examiner can be reached Monday-Friday from about 7:30 A.M. to about 7:00 P.M. Any inquiry of a general nature or relating to the status of this application should be directed to the Group receptionist whose telephone number is (703) 308-0196.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

H.J.Lilling: HJL
(571) 272-0918
Art Unit **1657**
January 08, 2008



Dr. Herbert J. Lilling
Primary Examiner
Group 1600 Art Unit 1657

is reminiscent of vesicles. Distinction between these models will rely on the identification of binding partners for Scrib. □

Methods

Fly stocks and cloning

scrib was mapped through assays of the ability of Y; autosome translocation chromosomes to provide paternal rescue of the *scrib* cuticle phenotype. About 15% of gametes from *T(Y;3)B158* males did not rescue the *scrib* germline clone (GLC) phenotype, whereas all gametes from *T(2;3)8r9* males did. This analysis limited the *scrib* region to 97BC, and a deficiency within this region (*Df(3R)Tl-x*) failed to complement *scrib*.

Non-complementing mutations were obtained from several collections; these new *scrib* alleles included two P-element-induced alleles. lethal phase analysis of the mutations and *Df(3R)Tl-x* indicates that the ethylmethane sulphonate–induced alleles *scrib*^{1} (formerly *7c10*; D.B. and N.P., unpublished data) and *scrib*^{2} (formerly *l(3)673*; K. Anderson, unpublished data) act as genetic nulls. *scrib j7B3* and *scrib s42405*[23,24] are induced by *P(lacW)* insertion. *scrib*^{1} and *scrib*^{2} were recombined onto *FRT82B* chromosomes for GLC production. The A2 cDNA was isolated from an embryonic cDNA library[25], and cloned into the vector pCasperhs for transgenic rescue experiments. For ectopic production of Crb, the strains *daGAL4* and *UAS-crb*^{1} were crossed at 29 °C.

Antibody production and embryonic analysis

Peptides corresponding to N-terminal (RYSRTLEELFLDANHIRDLPKNF) and C-terminal (VDAEDMRNPLDEIEAVEFRS) portions of Scrib were used to immunize rabbits (QCB, Hopkinton, MA). An affinity-purified serum gave similar staining through mid-embryogenesis to that seen with the unpurified sera used here; this staining is absent in *scrib* embryos. Western blots were carried out using standard techniques with 100 embryos from 0–2-h collections of *y w* or *scrib* GLCs. Antibodies were obtained as follows: anti-α-Spectrin from D. Kiehart; anti-Sas from D. Cavener; anti-Dlt, anti-Neurexin and anti-Crb from M. Bhat; anti-Cor from R. Fehon; anti-Ecad from H. Oda; anti-Neurotactin, anti-FasIII and anti-Arm from the Developmental Studies Hybridoma Bank. Secondary antibodies were from Jackson Labs. Fixation and staining was done as described[26], except that 5′ fixation periods were used for anti-Scrib staining. Embryos were staged according to standard methods[27], and assayed for polarization prior to stage 14. Fluorescent images were collected on a Leica TCS confocal microscope. Scanning electron micrographs were performed as described[28].

Received 15 October; accepted 12 August 1999.

1. Wodarz, A., Hinz, U., Engelbert, M. & Knust, E. Expression of Crumbs confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*. *Cell* **82**, 67–76 (1995).
2. Bhat, M. A. *et al.* Discs Lost, a novel multi-PDZ domain protein, establishes and maintains epithelial polarity. *Cell* **96**, 833–845 (1999).
3. Bryant, P. J. Junction genetics. *Dev. Genet.* **20**, 75–90 (1997).
4. Sieburth, D. S., Sun, Q. & Han, M. SUR-8, a conserved Ras-binding protein with leucine-rich repeats, positively regulates Ras-mediated signaling in *C. elegans*. *Cell* **94**, 119–130 (1998).
5. Suzuki, N. *et al.* Leucine-rich repeats and carboxyl terminus are required for interaction of yeast adenylate cyclase with RAS proteins. *Proc. Natl Acad. Sci. USA* **87**, 8711–8715 (1990).
6. Songyang, Z. *et al.* Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science* **275**, 73–77 (1997).
7. Nagase, T., Seki, N., Tanaka, A., Ishikawa, K. & Nomura, N. Prediction of the coding sequences of unidentified human genes. IV. The coding sequences of 40 new genes (KIAA0121–KIAA0160) deduced by analysis of cDNA clones from human cell line KG-1. *DNA Res.* **2**, 167–174 (1995).
8. Tepass, U. Epithelial differentiation in *Drosophila*. *BioEssays* **19**, 673–682 (1997).
9. Tepass, U., Theres, C. & Knust, E. Crumbs encodes an EGF-like protein expressed on apical membranes of *Drosophila* epithelial cells and required for organization of epithelia. *Cell* **61**, 787–799 (1990).
10. Brand, A. H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401–415 (1993).
11. Grawe, F., Wodarz, A., Lee, B., Knust, E. & Skaer, H. The *Drosophila* genes *crumbs* and *stardust* are involved in the biogenesis of adherens junctions. *Development* **122**, 951–955 (1996).
12. Cox, R. T., Kirkpatrick, C. & Peifer, M. Armadillo is required for adherens junction assembly, cell polarity, and morphogenesis during *Drosophila* embryogenesis. *J. Cell Biol.* **134**, 133–148 (1996).
13. Tepass, U. Crumbs, a component of the apical membrane, is required for zonula adherens formation in primary epithelia of *Drosophila*. *Dev. Biol.* **177**, 217–225 (1996).
14. Pawson, T. & Scott, J. D. Signaling through scaffold, anchoring, and adaptor proteins. *Science* **278**, 2075–2080 (1997).
15. Balda, M. S. & Matter, K. Tight junctions. *J. Cell Sci.* **111**, 541–547 (1998).
16. Dragsten, P. R., Blumenthal, R. & Handler, J. S. Membrane asymmetry in epithelia: is the tight junction a barrier to diffusion in the plasma membrane? *Nature* **294**, 718–722 (1981).
17. van Meer, G. & Simons, K. The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells. *EMBO J.* **5**, 1455–1464 (1986).
18. Jou, T. S., Schneeberger, E. E. & Nelson, W. J. Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases. *J. Cell Biol.* **142**, 101–115 (1998).
19. Yeaman, C., Grindstaff, K. K. & Nelson, W. J. New perspectives on mechanisms involved in generating epithelial cell polarity. *Physiol. Rev.* **79**, 73–98 (1999).
20. Grindstaff, K. K. *et al.* Sec6/8 complex is recruited to cell–cell contacts and specifies transport vesicle delivery to the basal-lateral membrane in epithelial cells. *Cell* **93**, 731–740 (1998).
21. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A. & von Zastrow, M. A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β2-adrenergic receptor. *Nature* **401**, 286–290 (1999).
22. Fernandez-Larrea, J., Merlos-Suarez, A., Urena, J. M., Baselga, J. & Arribas, J. A role for a PDZ protein in the early secretory pathway for the targeting of proTGF-α to the cell surface. *Mol. Cell* **3**, 423–433 (1999).
23. Spradling, A. C. *et al.* The Berkeley *Drosophila* genome project gene disruption project. Single P-element insertions mutating 25% of vital drosophila genes. *Genetics* **153**, 135–177 (1999).
24. Salzberg, A. *et al.* P-element insertion alleles of essential genes on the third chromosome of *Drosophila melanogaster*: mutations affecting embryonic PNS development. *Genetics* **147**, 1723–1741 (1997).
25. Brown, N. H. & Kafatos, F. C. Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* **203**, 425–437 (1988).
26. Bilder, D., Graba, Y. & Scott, M. P. Wnt and TGFβ signals subdivide the AbdA Hox domain during *Drosophila* mesoderm patterning. *Development* **125**, 1781–1790 (1998).
27. Campos-Orgega, J. A. & Hartenstein, V. *The Embryonic Development of Drosophila melanogaster* (Springer, Heidelberg, 1997).
28. Hacker, U. & Perrimon, N. DRhoGEF2 encodes a member of the Dbl family of oncogenes and controls cell shape changes during gastrulation in *Drosophila*. *Genes Dev.* **12**, 274–284 (1998).
29. Apperson, M. L., Moon, I. S. & Kennedy, M. B. Characterization of Densin-180, a new brain-specific synaptic protein of the O-sialoglycoprotein family. *J. Neurosci.* **16**, 6839–6852 (1996).

Acknowledgements

We would like to thank E. Knust, M. Bhat, D. Kiehart, R. Fehon, D. Cavener, H. Oda and K. Anderson for providing antibodies and fly stocks. We are indebted to Min Li for excellent technical assistance and B. Mathey-Prevot, M. Petitt, and I. The for comments on the manuscript. D.B. is an American Cancer Society Fellow. N.P. is an Investigator of the Howard Hughes Medical Institute.

Correspondence and requests for materials should be addressed to D.B. (e-mail: bilder@rascal.med.harvard.edu).

A tripeptide 'anticodon' deciphers stop codons in messenger RNA

Koichi Ito, Makiko Uno & Yoshikazu Nakamura

Department of Tumor Biology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

The two translational release factors of prokaryotes, RF1 and RF2, catalyse the termination of polypeptide synthesis at UAG/UAA and UGA/UAA stop codons, respectively[1–3]. However, how these polypeptide release factors read both non-identical and identical stop codons is puzzling[4]. Here we describe the basis of this recognition. Swaps of each of the conserved domains between RF1 and RF2 in an RF1–RF2 hybrid led to the identification of a domain that could switch recognition specificity. A genetic selection among clones encoding random variants of this domain showed that the tripeptides Pro-Ala-Thr and Ser-Pro-Phe determine release-factor specificity *in vivo* in RF1 and RF2, respectively. An *in vitro* release study of tripeptide variants indicated that the first and third amino acids independently discriminate the second and third purine bases, respectively. Analysis with stop codons containing base analogues indicated that the C2 amino group of purine may be the primary target of discrimination of G from A. These findings show that the discriminator tripeptide of bacterial release factors is functionally equivalent to that of the anticodon of transfer RNA, irrespective of the difference between protein and RNA.

A tRNA-like property has been speculated for release factors[5,6] in reading stop codons, and our aim was to identify a putative peptide anticodon equivalent in release factors. From sequence comparisons, we have previously proposed a seven-domain structure for release factors (domains A–G; Fig. 1a, top). We swapped these seven domains combinatorially between RF1 (UAG-specific) and RF2 (UGA-specific) to screen for active release-factor hybrids that would display altered codon specificity *in vivo*. For this purpose, common restriction sites were introduced into a clone at, or near, the sequences encoding the domain junctions so that these base changes would not affect activity (Fig. 1a, bottom). We examined a combinatory set of 128 release-factor hybrids for their ability

to complement RF1 knockout (*prfA*::Km^R) and RF2 knockout (*prfB*::Cm^R) alleles (see Methods). For the initial screening, phenotypic assessment of RF1 and RF2 activity was more reliable than *in vitro* analysis because of the difference in their specific activities and the occurrence of peptide-anticodon-independent omnipotent variants that recognise all three stop codons[7].

These release-factor hybrids were scored in pair-wise combinations in which only one domain differed. The criterion used to assign the discriminator domain was its ability to switch, without exception, the complementation activity exclusively and efficiently between RF1 and RF2. We found that a unique set of functional release-factor hybrids, designated ΨRF1 and ΨRF2, containing a single distinct domain, domain D (Fig. 1b), showed RF1- and RF2-specific *in vivo* complementation (see Fig. 2b) and *in vitro* peptide-release activities (see Fig. 3a, b). Any active release-factor hybrid other than these two switched the selectivity upon substituting domain D between RF1 and RF2 (data not shown). We concluded that domain D contains the 'peptide anticodon'. We also concluded that the hybrid-release-factor sequence, referred to as ΨRF (see Fig. 1b, bottom), can play a vital role in the assessment of the discriminator activity upon swapping the peptide anticodon segment between RF1 and RF2. The native RF1 and RF2 sequences were simply inactivated by the same swap (data not shown). Switching the RNA-recognition specificity with a peptide swap in tRNA synthetases works using a hybrid[8] or non-hybrid[9] construct depending on the enzymes.

The discriminator domain was further defined by swapping peptides within domain D. Construction of a series of intradomain release-factor hybrids permitted assignment of discriminator activity to the central 15-amino-acid segment located in one of the most conserved regions. Five out of six residues in this segment differ and are perfectly conserved in RF1s and RF2s of many species (Fig. 2a; see ref. 6). Transfer of a 24-residue segment containing these 15 amino acids from RF1 to ΨRF2 switched the specificity from RF2 to RF1 (ΨRF1β; Fig. 2a; see Fig. 3c). Subsequent experiments used variants of this 15-residue segment as discriminator cassettes in hybrid domain D (Fig. 1b, bottom). The conserved RF1- and RF2-specific motifs in this region were 'Q-vPaTE- - - -I' and 'V-kSPFD- - - -R', respectively, where lower case represents less conserved, and upper case represents highly conserved, residues (Fig. 2a). Primary screening of the requirements for the discriminator by site-directed and combinatory mutagenesis indicated that substitutions of amino acids other than 'PaT' and 'SPF' between these two motifs did not affect the selectivity of codons (data not shown). Hence, we used 'Q-VXXXD- - - -I' as a universal cassette motif, for which tripeptide XXX was extensively analysed.

The systematic selection of the discriminator motifs was carried out using designed tripeptide collections in which the first or third position was fixed as an amino acid of RF1 and RF2 in otherwise (partly) random sequences (see Fig. 2a, bottom): PX_4X_{20} (pool-1), SX_4X_{20} (pool-2), $X_{20}X_4T$ (pool-3), $X_{20}X_4F$ (pool-4), and $SX_{20}T$ (pool-5), where P = Pro, S = Ser, T = Thr, F = Phe, X_4 = Ala/Pro/Ser/Thr, and X_{20} = 20 amino acids (Table 1). Winners from pool-1 in the RF1-null strain contained any of four residues at the second position but mostly threonine (56 out of 78). Less frequently, other non-charged amino acids were at the third position, and no winners appeared in the RF2-null strain. Winners from pool-2 in RF1 and RF2-null strains contained proline at the second position (note that alanine and serine were also allowed in RF1) and mostly shared

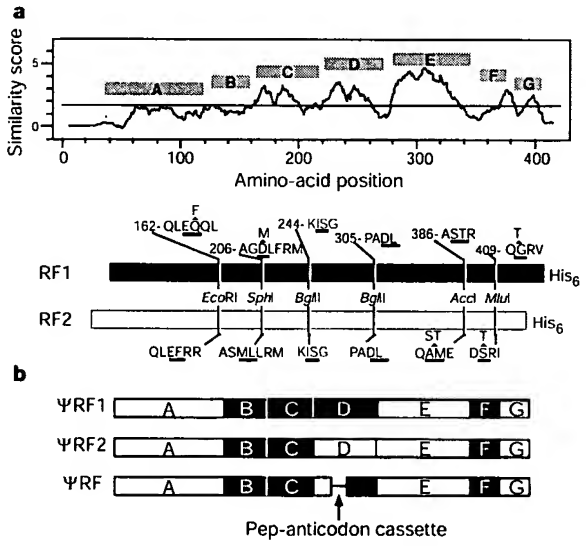


Figure 1 A seven-domain model of release factors and release-factor hybrids developed for the pep-anticodon assignment. **a**, The average similarity plots of 22 bacterial RF1 and RF2 sequences and the proposed seven conserved domains A–G (ref. 6). Restiction-enzyme sites were generated at or near the domain junctions whose relevant peptide sequences are shown. Amino acids for which restriction sites were designed are underlined: most of the nucleotide-sequence changes did not alter amino acids except in five positions (changes are marked by arrows) that altered single (non-conserved) residues. The amino-acid position refers to the coordinate of the similarity alignment (ref. 6), which is distinct from those of native RF1 and RF2. **b**, A pair of release-factor hybrids, ΨRF1 and ΨRF2, that exerted RF1- and RF2-specific complementation activities owing to the respective domain D inserts. A hybrid release-factor construct, designated ΨRF, was used as a test release-factor backbone to monitor the specificity of transplanted peptides and an amino-acid swap made in the discriminator tripeptide. Closed and open boxes represent RF1 and RF2 segments, respectively.

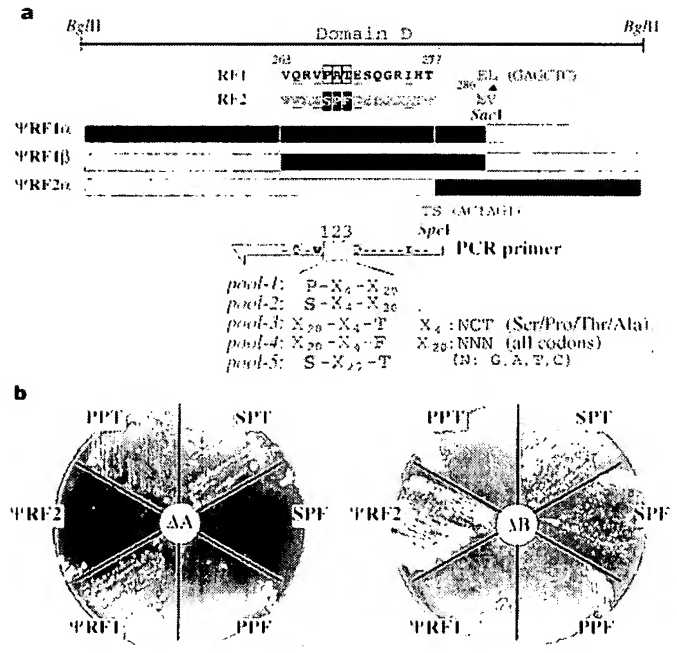


Figure 2 The assessment of the discriminator tripeptide within domain D. **a**, Intradomain D hybrids that exerted RF1- and RF2-specific complementation activities owing to the respective mosaic intervals. These hybrid sequences were transplanted into the ΨRF construct and examined for their specificity by *in vivo* complementation and *in vitro* release assay. The amino-acid sequence of the assigned discriminator locus is shown. The discriminator tripeptides are boxed and other conserved residues are underlined. Restriction sites for peptide transplantation are shown with the relevant dipeptide (arrow indicates that the amino acid is changed). PCR primers designed for random pools of the discriminator tripeptides are indicated (see Table 1). **b**, Growth of RF1-null (ΔA, RM789A; left panel) and RF2-null (ΔB, RM789B; right panel) strains transformed with pBAX-RF derivatives carrying: ΨRF1, ΨRF2, PPT, SPT, SPF and PPF tripeptide variations.

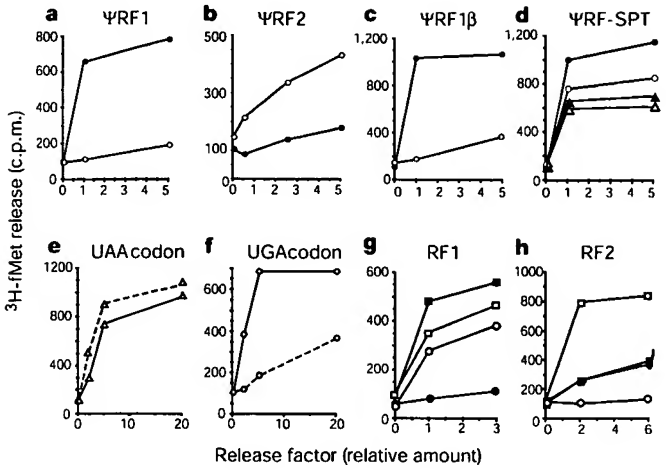


Figure 3 Polypeptide release activity of the discriminator variants and inosine variants of stop triplets. ^{3}H-fMet release from the ^{3}H-fMet-tRNA^f-mini-messenger-ribosome complex upon addition of release factors was determined[14]. The same molar amount of each release factor variant relative to ΨRF1 was used. Mini-messenger RNAs used in these reactions were 9-nucleotide sequences consisting of 5′-UUCAUG-3′ followed by stop (or test) triplets. **a**, ΨRF1. **b**, ΨRF2. **c**, ΨRF1β. **d**, ΨRF-SPT variant. Test triplets of the mini-messengers (20 μM) in reactions shown in **a**–**d** were UAG (filled circles), UGA (open circles), UAA (open triangles) and UGG (filled triangles). **e**, UAA termination assay of SPF (solid line) and PPF (dashed line) variants. **f**, UGA termination assay of SPF (solid line) and PPF (dashed line) variants. Reactions shown in **g** and **h** contained inosine-substituted stop triplets in mini-messengers: UAI (filled squares), UIA (open squares), UGI (filled circles) and UIG (open circles). **g**, RF1. **h**, RF2. Experiments were carried out independently at least three times, and the mean values are expressed. The experimental errors are less than 10%.

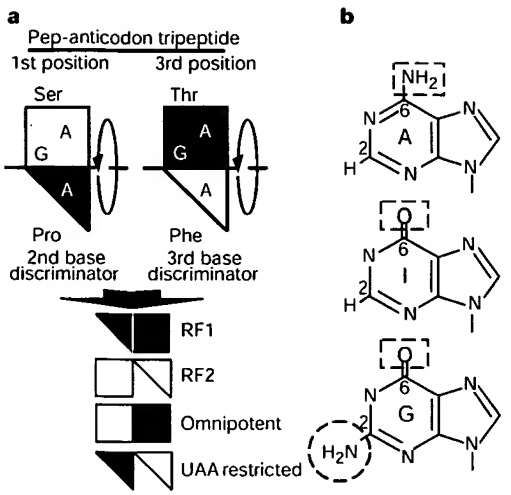


Figure 4 The discriminator amino acids of pep-anticodon tripeptides and their target group of purine bases. **a**, The double-switch model for the discriminator tripeptide. Second and third base discrimination is carried out by first and third amino acids of the discriminator tripeptide, respectively: Amino acids next to squares are permissive to A and G, whereas those next to triangles are restrictive to A. Circular arrows indicate that these discriminator amino acids can be switched. Omnipotent and UAA-restricted variants were made in this study. **b**, The defined discriminated group(s) in purine bases. Inosine-substitution experiments indicated that the C2 amino group of G is a primary discriminator target. The C6 carbonyl and amino groups in G and A also weakly affect discrimination.

amino acids at the third position (these tripeptides acquired omnipotence for three stop codons as shown below) except for phenylalanine which was restricted to the RF2-null strain. Winners from pool-3 in the RF1-null strain contained serine or proline at the first position and any four residues at the second position, and the only winner in the RF2-null strain was Ser-Pro-Thr (omnipotent, see below). When the third position is fixed as phenylalanine (pool-4), the sole winner was Ser-Pro-Phe which appeared in the RF2-null strain. Growth of the RF1- or RF2-null strains in the presence of representative tripeptide variants, PPT (RF1-type), SPT (omnipotent), SPF (RF2-type) and PPF (see below), is shown in Fig. 2b. These and other *in vivo* complementation data are summarized in Table 1.

The above genetic selection determined the tripeptide amino acids, including both conserved and non-conserved residues, that are required for discriminator activity. We then focused on the tripeptides that consist of the natural amino acids used in RF1 and RF2 to dissect individual amino-acid function using the *in vitro* release assay. Of the tripeptides selected using RF1- or RF2-null conditions, several such as Ser-Pro-Thr and Ser-Pro-Arg acquired omnipotent activity and were able to complement the RF1–RF2 double knockout strain. Consistently, the Ser-Pro-Thr variant catalysed polypeptide release at all three stop codons, UAA, UAG and UGA (Fig. 3d). In spite of the apparent complexity of the tripeptide sequence variations, these patterns can be easily understood by assuming that the first and third amino acids of the tripeptide discriminate the second and third bases of the stop codon, respectively, and that these two discriminators can operate independently ('double-switch model'; Fig. 4a). Thus, at the first position, proline is restrictive to A (RF1), while Ser is permissive to both A and G (RF2). At the third position, threonine is permissive to A and G (RF1), whereas phenylealanine is restrictive to A (RF2). Pool-1 selection data indicated that the second position of the tripeptide in RF1 is less restrictive, and that proline is effective, hence further manipulations of the tripeptide included proline at the second position (as shown in Fig. 3e, f). The double-switch model for stop codon discrimination is validated and clarified further by two lines of evidence. First, the substitution of Pro-Pro-Phe for Ser-Pro-Phe in an otherwise ΨRF2 backbone reduced UGA termination but had virtually no effect on UAA termination, even with a large amount of this variant (compare Fig. 3e, f). Second, the omnipotent tripeptide Ser-Pro-Thr also conferred peptide release activity with UGG as potential stop codon (Fig. 3d). These results led us to conclude that the defined tripeptide represents the discriminator element of the peptide anticodon; hereafter referred to as 'pep-anticodon'. We believe that this is a naturally functioning pep-anticodon, not a hybrid-specific one, because of the hybrid context and because the tripeptide is made up entirely of the sequences of the natural release factors.

Compared with the variation of tripeptide winners in RF1-null strains, winners in RF2-null strains were restricted to tripeptides having serine and proline at the first and second positions, respectively. This finding indicates that there may be biased structural requirement(s) in the discriminator tripeptide for the relaxed recognition of both A and G at the second base in contrast to the third base. Consistent with this hypothesis, when tripeptide pool-5 ($SX_{20}T$) was used for selection, a winner in the RF2-null strain contained a single residue, proline (omnipotent), whereas those in RF1-null strains allowed 15 other effective residues which were specific to UAA/UAG (they did recognise UGA) (Table 1).

What atoms or atomic groupings of purine bases are discriminated by the discriminator tripeptide? In principle, two side groups may be potent targets, either C2 amino (G) discriminated from C2 proton (A) or C6 carbonyl (G) discriminated from C6 amino (A), or both (Fig. 4b). To test these possibilities, we substituted inosine (I; see Fig. 4b) for G in the stop codon and analysed polypeptide

Table 1 Selection of discriminator tripeptides of pep-anticodon

Selected pool	Winners in RF1-null strains (score/specificity)		Winners in RF2 null-strains (score/specificity)	
Pool-1	(78 clones scored)		(no clones selected)	
PX_4X_{20}	PTT (20/A) PST (12/A) PPN (8/A) PSN (3/A) PTS (2/A) PSQ (1/A)	PAT (16/A) PPT (8/A) PTN (3/A) PPS (3/A) PPL (2/A)	(none)	
Pool-2	(50 clones scored)		(49 clones scored)	
SX_4X_{20}	SPT (19/AB) SPL (7/AB) SPI (1/AB) SST (1/AB)	SPR (19/AB) SAT (2/AB) SPH (1/AB)	SPT (19/AB) SPI (7/AB) SPL (2/AB) SPH (1/AB)	SPR (16/AB) SPF (3/B) SPM (1/AB)
Pool-3	(68 clones scored)		(68 clones scored)	
$X_{20}X_4T$	SPT (18/AB) SAT (13/A) PTT (8/A) STT (2/A)	PST (14/A) PPT (9/A) PAT (4/A)	SPT (68/AB)	
Pool-4	(no clones selected)		(34 clones scored)	
$X_{20}X_4F$	(none)		SPF (34/B)	
Pool-5	(81 clones scored)		(41 clones scored)	
$SX_{20}T$	SPT (19/AB) SRT (8/A) STT (6/A) SST (5/A) SVT (3/A) SGT (3/A) SNT (2/A) SDT (1/A)	SAT (9/A) SLT (7/A) SHT (6/A) SIT (4/A) SKT (3/A) SQT (2/A) SFT (2/A) SMT (1/A)	SPT (41/AB)	

ΨRF variants carrying designed (random) sequence pools of discriminator tripeptides were transformed into RF1-null and RF2-null strains, and active variants were selected (see Methods). These winners were examined for their sequence and activity to complement RF1- and/or RF2-null mutations. The number of winners scored and the specificity are shown in parentheses. Specificity: A, RF1 specificity (*prfA* complementation); B, RF2 specificity (*prfB* complementation); AB, omnipotent specificity (*prfAB* double mutant complementation). Amino acids are in single-letter code. X_4 represents Ala/Pro/Ser/Thr; and X_{20} represents 20 amino acids.

release *in vitro*. Both UAI and UIA were recognized by RF1 and RF2 (Fig. 3g, h), showing that the inosine substitution removed the primary discrimination target. On the other hand, UIG and UGI were still recognized selectively by RF1 and RF2, respectively (Fig. 3g, h). Therefore, we believe that the pep-anticodon discriminates primarily the C2 amino group of G at both second and third bases. Nevertheless, the potential contribution, if any, of the C6 amino group of A remains to be examined because of the apparently lower release activity of A-to-I variants at the discriminator position (see Fig. 3g, h).

The principal rule of base discrimination by the discriminator tripeptide is that amino acids used for restricted recognition of A are bulky hydrophobic residues, such as proline and phenylalanine, whereas those for relaxed recognition of A and G are small hydrophilic residues, such as serine and threonine. The latter, relaxed recognition of A and G may be phenotypically equivalent to wobble pairing between the messenger RNA triplet and tRNA anticodon at the third position[10,11]. What is unique to release factors is allowing wobble not only at the third position of the stop codon but also at the second position. It is of interest that in eukaryotes a single release factor, eRF1, recognizes all three stop codons. An omnipotent discriminator tripeptide, such as Ser-Pro-Thr, cannot account for the three-codon-specific recognition because it recognizes UGG as well. Therefore, eRF1 must have its own discrimination mechanism: the simplest way to achieve this would be to permit two purine bases to exist at the second and third positions, except for double G, in a single recognition site. Our identification of the discriminator tripeptide strongly suggests that release factors recognize stop codons directly rather than indirectly through the interplay of stop codons and rRNA sequences[12]. The amino acid(s) responsible for recognition of the first base, U, of the stop codon must be the adjacent residue(s) conserved in both RF1 and RF2. In summary, our discovery of the discriminator tripeptide, or pep-anticodon, in bacterial release factors clearly solves the long-standing coding problem of how a release factor reads a stop codon. Moreover, it is important to point out that our experimentally defined discriminator tripeptide agrees with the predictions of our release-factor tRNA mimicry hypothesis[2,6]. □

Methods

Plasmids and manipulations

Plasmid pBAX-RF is a pBR322-derived RF expression plasmid[7] in which release-factor hybrids or those with domain cassettes are inserted into *Bam*HI–*Sac*I (replaced for *Nru*I) sites and expressed constitutively from the *tet* promoter. The release-factor hybrids contained a carboxy-terminal histidine tag encoded by a sequence generated by PCR primers as described[13]. Intervals of domains A–G of RF1 and RF2 were marked with restriction enzyme sites using designed PCR primers (see Fig. 1a). These artificial restriction sequences did not affect release-factor activity. Interdomain swapping was carried out by amplification of the appropriate DNAs from RF1 and RF2, followed by substitution of their restriction fragments. Intradomain swapping was achieved using PCR primers that were designed to encode hybrid release-factor sequences.

In vivo complementation and selection

Escherichia coli W3110 derivatives[7,14], RM789A (*prfA*::Km^R), RM789B (*prfB*::Cm^R) and RM789AB (*prfA*::Km^R *prfB*::Cm^R), carrying plasmid pSUIQT-RF2*[7], were used as indicator hosts. pSUIQT-RF2* is a *lac* promoter-controlled expression plasmid of an RF2 variant, RF2*, that is able to recognize UAA/UGA/UAG; hence, it complements the single or double RF1–RF2 knockout alleles in the presence of isopropyl-1-thio-β-D-galactoside (IPTG). For complementation tests, indicator strains were transformed with two compatible plasmids, pSUIQT-RF2* and a test pBAX-RF construct, in the presence of IPTG, and growth was monitored in the absence of IPTG. For selections, pSUIQT-RF2*-bearing indicator cells were transformed with pBAX-RF hybrid or cassette pools, and survivors were selected in the absence of IPTG to give rise to pBAX-RF winners. These results were consistent with those derived from temperature-sensitive alleles in RF1 (*prfA1*)[15] and RF2 (*prfB286*)[16] (data not shown).

Peptide pools

Randomized collections of the discriminator tripeptide encoded in 76-mer primers (antisense; 5′-GGGGATCCACTAGTATGAATGCGACCCTGGGAGTCNNNCGNNN-NAACACGTTGCACACGATGTACGCCGGTTTCAG-3′ where N indicates A/G/T/C mix) carrying designed sequence variations in domain D were used to amplify ΨRF segments containing domains A-D by PCR using the 5′ flanking vector sequence (5′-CGAC-

TACGCGATCATGGCGAC-3'). The amplified DNA pools were digested with *Bam*HI and *Spe*I restriction enzymes, and inserted into the tester plasmid pBAX-ΨRF (see Fig. 2a, bottom).

Protein purification and peptide release assay

Histidine-tagged release-factor genes were cloned downstream of a T7 RNA polymerase promoter in plasmid pET30a (Novagen, Inc.), and overexpressed in *E. coli* BL21 (DE3) cells by addition of 0.5 mM IPTG for 2.5 h as described[7,13,14]. Histidine-tagged release-factor proteins were purified to homogeneity from cell lysates by affinity chromatography using Ni-NTA Agarose (Qiagen). The ability of purified release-factor hybrids to terminate protein synthesis was monitored by the rate of *N*-formylmethionine (fMet) release at the stop codon from the *in vitro* termination complex composed of ^{3}H-fMet-tRNA^{f}, 9-nucleotide mini-messenger (5'-UUCAUGNNN-3' where NNN is a test triplet) and the ribosome isolated from *E. coli* essentially as described[7,14].

Received 16 August; accepted 18 November 1999.

1. Scolnick, E., Tompkins, R., Caskey, T. & Nirenberg, M. Release factors differing in specificity for terminator codons. *Proc. Natl Acad. Sci. USA* **61**, 768–774 (1968).
2. Nakamura, Y., Ito, K. & Isaksson, L. A. Emerging understanding of translation termination. *Cell* **87**, 147–150 (1996).
3. Buckingham, R. H., Grentzmann, G. & Kisselev, L. Polypeptide chain release factors. *Mol. Microbiol.* **24**, 449–456 (1997).
4. Nakamura, Y. & Ito, K. How protein reads the stop codon and terminates translation. *Genes Cells* **3**, 263–278 (1998).
5. Moffat, J. G. & Tate, W. P. A single proteolytic cleavage in release factor 2 stabilizes ribosome binding and abolishes peptidyl–tRNA hydrolysis activity. *J. Biol. Chem.* **269**, 18899–18903 (1994).
6. Ito, K., Ebihara, K., Uno, M. & Nakamura, Y. Conserved motifs of prokaryotic and eukaryotic polypeptide release factors: tRNA–protein mimicry hypothesis. *Proc. Natl Acad. Sci. USA* **93**, 5443–5448 (1996).
7. Ito, K., Uno, M. & Nakamura, Y. Single amino acid substitution in prokaryote polypeptide release factor 2 permits it to terminate translation at all three stop codons. *Proc. Natl. Acad. Sci. USA* **95**, 8165–8169 (1998).
8. Auld, D. S. & Schimmel, P. Switching recognition of two tRNA synthetases with an amino acid swap in a designed peptide. *Science* **267**, 1994–1996 (1995).
9. Wakasugi, K., Quinn, C. L., Tao, N. & Schimmel, P. Genetic code in evolution: switching species-specific aminoacylation with a peptide transplant. *EMBO J.* **17**, 297–305 (1998).
10. Crick, F. H. C. Codon–anticodon pairing: the wobble hypothesis. *J. Mol. Biol.* **19**, 548–555 (1966).
11. Soll, D. & RajBhandary, U. L. Studies on polynucleotides. LXXVI. Specificity of tRNA for codon recognition as studied by amino acid incorporation. *J. Mol. Biol.* **29**, 113–124 (1967).
12. Murgola, E. J., Hijiazi, K. A., Goringer, H. U. & Dahlberg, A. E. Mutant 16S rRNA: a codon specific translational suppressor. *Proc. Natl Acad. Sci. USA* **85**, 4162–4165 (1988).
13. Uno, M., Ito, K. & Nakamura, Y. Functional specificity of amino acid at position 246 in the tRNA mimicry domain of bacterial release factor 2. *Biochimie* **78**, 935–943 (1996).
14. Yoshimura, K., Ito, K. & Nakamura, Y. Amber (UAG) suppressors affected in UGA/UAA-specific polypeptide release factor 2 of bacteria: genetic prediction of initial binding to ribosome preceding stop codon recognition. *Genes Cells* **4**, 253–266 (1999).
15. Rydén, S. M. & Isaksson, L. A. A temperature-sensitive mutant of *Escherichia coli* that shows enhanced misreading of UAG/A and increased efficiency for some tRNA nonsense suppressors. *Mol. Gen. Genet.* **193**, 38–45 (1984).
16. Kawakami, K., Inada, T. & Nakamura, Y. Conditionally lethal and recessive UGA–suppressor mutations in the *prfB* gene encoding peptide chain release factor 2 of *Escherichia coli*. *J. Bacteriol.* **170**, 5378–5381 (1988).

Acknowledgements

We thank C. Yanofsky and J. Atkins for critical reading of the manuscript and valuable comments. This work was supported in part by grants from The Ministry of Education, Science, Sports and Culture, Japan; the Human Frontier Science Program (awarded in 1993 and 1997); and the Basic Research for Innovation Biosciences Program of Bio-oriented Technology Research Advancement Institution (BRAIN).

Correspondence and requests for materials should be addressed to Y.N. (e-mail: nak@ims.u-tokyo.ac.jp).

Identification and Synthesis of Chemotactic Tripeptides From Alkali-Degraded Whole Cornea

A Study of N-acetyl-Proline-Glycine-Proline and N-methyl-Proline-Glycine-Proline

Roswell R. Pfister, Jeffrey L. Haddox,* Charnell I. Sommers,* and Kwok-Wai Lam†*

Purpose. To identify and synthesize the polymorphonuclear leukocyte chemoattractant(s) released from alkali-degraded corneas.

Methods. Corneas were degraded in 1.0 N NaOH, neutralized, ultrafiltered, and dialyzed. The final active ultrafiltrate was subjected to high-performance liquid chromatography on a Protein PAK I-60 column. The most active fractions were further separated on a μ-Bondapak-C_{18} and I-60 column in sequence.

Results. Fraction 38 from the final I-60 column associated with a 210-nm absorption peak and elicited a polarization and chemotactic response from polymorphonuclear leukocytes. The loss of polarization activity in fraction 38 after exposure to prolidase suggests that this peptide contains a Pro-X (X = amino acid) peptide bond. The amino acid composition of fraction 38 was 35% glycine and 53% proline. Peptide sequence analysis was unable to establish a primary sequence even though Picotag analysis showed the presence of large amounts of the two amino acids. Mass spectrometry revealed only two molecular species of 312 MWt and 284 MWt. Tripeptides were synthesized using all possible amino acid permutations of 2 Pro and 1 Gly and tested in the polarization and chemotactic assays. These techniques demonstrated that n-acetyl-Pro-Gly-Pro, and to a lesser degree n-methyl-Pro-Gly-Pro, were the only synthetic tripeptides with activity similar to the purified chemoattractant.

Conclusions. The data show that the chemotactic peptides, purified from alkali-degraded whole cornea and confirmed with identical synthetic tripeptides, are N-acetyl-Pro-Gly-Pro and N-methyl-Pro-Gly-Pro. Although a number of proteins contain the Pro-Gly-Pro sequence, large amounts of collagen in the cornea suggest this as a major source. The small size and hydrophilic nature of these chemoattractants are predictive of a high degree of diffusibility. These chemoattractants are likely to play a major role in the early neutrophil response after an alkali injury. Invest Ophthalmol Vis Sci. 1995;36:1306–1316.

Severe alkali injury to the eye results in vigorous infiltration of inflammatory cells into the cornea.[1-8] The predominant cell type found is the polymorphonuclear leukocyte (PMNL), usually associated with the development of corneal ulcers. Polymorphonuclear leukocyte accumulation is generally accepted to result from the directional migration of cells in response to a chemoattractant gradient. The mediators that trigger the chemotactic response, therefore, are crucial to initiating the inflammatory response. Knowledge of the type of mediators released that stimulate the acute inflammatory response in the alkali-injured cornea is important to understanding the molecular mechanism of ulceration and to developing rational therapeutic regimens.

We have shown[9,10] that mediators of PMNL activity are generated directly by alkali degradation of whole cornea. Our initial studies showed that alkali digestion of cornea in vitro generated a stimulant of the PMNL

*From *The Eye Research Laboratories, Brookwood Medical Center, Birmingham, Alabama, and the †University of Texas Health Sciences Center, San Antonio, Texas.*
Supported by the National Eye Institute grant EY04716.
Submitted for publication June 10, 1994; revised January 13, 1995; accepted January 17, 1995.
Proprietary interest category: N.
Reprint requests: Roswell R. Pfister, The Eye Research Laboratories, Brookwood Medical Center, 2008 Brookwood Medical Center Drive, Suite 211, Birmingham, AL 35209.

Investigative Ophthalmology & Visual Science, June 1995, Vol. 36, No. 7

respiratory burst and activated PMNL migration as measured by the Boyden chamber. Polymorphonuclear locomotion in the Boyden chamber studies was probably caused by the presence of a chemoattractant(s), but the technique was not sufficient to measure chemotaxis. Our more recent development of a collagen gel–visual chemotactic assay system permitted direct measurement of chemotaxis and cellular behavior in a simulated physiological environment. These studies revealed true PMNL chemotaxis in the low molecular weight ultrafiltrate.[11]

The purpose of the current study was to isolate, characterize, identify, and synthesize the low molecular weight PMNL chemoattractant(s) released from alkali-degradation of whole cornea. An additional aim was to determine if the purified and synthetic chemoattractants elicit a chemotactic response from PMNLs.

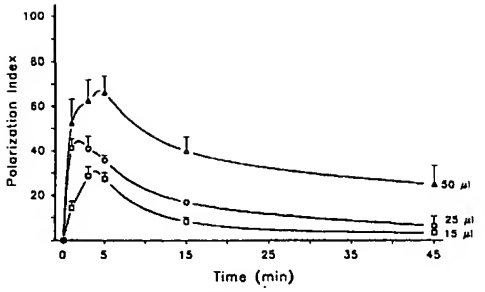


FIGURE 1. Time–response curves for the polarization index of polymorphonuclear leukocytes exposed to three doses of the 100- to 1000-MWt ultrafiltrate. The negative control value (polymorphonuclear leukocytes in buffer only) was subtracted from the total polarization score to achieve a polarization index for each sample. Fifteen microliters (*squares*), 25 µl (*circles*), or 50 µl (*triangles*) of ultrafiltrate were added to each incubation mixture, producing a final volume of 250 µl. Each data point represents the mean ± standard error of the mean, $n = 5$.

MATERIALS AND METHODS

General

Hanks' balanced salt solution (HBSS) was purchased from Gibco Laboratories (Chagrin Falls, OH). Calcium chloride, $MgCl_2$, glutaraldehyde, sodium azide, Ficoll (type 400), and prolidase (proline dipeptidase, EC 3.4.13.9) were purchased from Sigma Chemical (St Louis, Mo). Sodium hydroxide, sodium phosphate monobasic, and sodium·phosphate dibasic were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium chloride was obtained from Mallinckrodt (Paris, KY). Hydrochloric acid was purchased from Ricca Chemical (Arlington, TX). Hypaque-76 was obtained from Winthrope Laboratories (New York, NY). Leukotriene B_4 (LTB_4) was a generous gift from Merck Frosst Canada (Point Claire–Dorval, Quebec, Canada).

Vitrogen 100 from Celtrix Laboratories (Palo Alto, CA) was used to prepare the collagen monomer solution for the collagen gel–visual chemotactic assay. The collagen solution consisted of 0.8 ml of Vitrogen 100 (2.5 mg collagen/ml; Celtrix), 0.1 ml of 0.1 N NaOH, and 0.1 ml of 10× phosphate-buffered saline (pH 7.3). This solution was mixed at 5°C and allowed to warm to room temperature immediately before use.

Polymorphonuclear Leukocyte Isolation

These experiments followed the tenets of the Declaration of Helsinki and were approved by the human research committee at Brookwood Medical Center. All donors signed written consent forms explaining the nature and possible consequences of the study. Blood was collected from only one donor each day. According to the technique of Ferrante and Thong,[12] PMNLs were isolated from fresh heparinized human blood by centrifugation on Hypaque–Ficoll (density = 1.114) according to a previous article.[13] Isolated PMNLs (96% to 99% viability) were resuspended in HBSS at room temperature and gently agitated on a shaker. The purity of this cell suspension was ≥85% PMNLs and ≤5% mononuclear cells and platelets, with the remaining percentage consisting of red blood cells. To measure the response of cells to chemoattractants, purified PMNLs were used in two separate systems, polarization and collagen gel–visual chemotactic assays. All incubation mixtures from both assays maintained an osmolality between 270 and 300, a pH range of 7.2 to 7.6, and a Ca^{2+} and Mg^{2+} concentration of 500 µM and 600 µM, respectively.

Polarization Assay

The polarization assay[14] was used as a preliminary screening test for large numbers of samples by quantitating the shape change that occurs after the exposure of PMNLs to a chemoattractant. Briefly, 1×10^6 PMNLs were suspended in HBSS and phosphate-buffered saline in incubation mixtures to produce a physiological osmolality and pH for each sample or high-performance liquid chromatography (HPLC) fraction to be tested. Each incubation mixture (total volume = 250 µl) was then exposed to a sample in a stirred reaction chamber at 35°C for 5 minutes, unless otherwise noted. At the end of the incubation period, each cell suspension was mixed with an equal volume of 4% glutaraldehyde. Polymorphonuclear leukocytes in each sample were observed microscopically and assigned scores of 0 (resting = spherical with a smooth membrane), 1 (activated = irregular with uneven membranes), or 2 (polarized = length ≥ width × 2).

TABLE 1. Polymorphonuclear Leukocyte Polarization Activity Generated by the Final HPLC Active Fraction 38 After Prolidase Treatment

Sample	Incubation Time (hours)	Polarization Score	Activity (%)
Positive Control			
75 μl 100–3,000	2	85	100
UF + HBSS	4	86	100
Negative Control			
75 μl HBSS +	2	2	0
prolidase–HBSS	4	2	0
Experimental			
75 μl 100–3,000 UF	2	34	39
+ prolidase–HBSS	4	12	12
Positive Control			
100 μl fraction			
38 + HBSS	4	63	100
Negative Control			
100 μl HBSS +			
prolidase–HBSS	4	1	0
Experimental			
100 μl fraction 38			
+ prolidase–HBSS	4	15	23

Each sample contained equal amounts of chemoattractant, enzyme and HBSS where appropriate. The final volume of each PMNL incubation mixture was 500 μl. The negative control value was subtracted from each sample, and polarization activity was expressed as a percentage of the positive control.
HPLC = high-performance liquid chromatography; UF = ultrafiltrate; HBSS = Hanks' balanced salt solution.

The scores of 100 PMNLs were added, producing a total polarization score. Negative control values (PMNLs in buffer only) were subtracted from all samples to achieve a polarization index (as in Fig. 1) or, in some cases, polarization activity was expressed as a percentage of the positive control (as in Table 1) or as specific polarization activity.

Collagen Gel–Visual Chemotactic Assay

The collagen gel–visual chemotactic assay (CG–VCA)[11,15,16] was used as the conclusive test for the presence of chemotactic activity. In this assay, the chemotactic movement of PMNLs through a collagen gel was measured directly by a computer-assisted visual tracking system. Immediately before the addition to the CG–VCA, the PMNL suspension was centrifuged at 60*g* for 4 minutes, and the collagen solution was simultaneously brought to room temperature. The PMNL pellet was gently resuspended in the collagen solution (2.0×10^7 PMNLs/ml), which was then added to the CG–VCA system and allowed to gel. Thick walled dialysis membrane (12,000 to 14,000 MWt cutoff pore size, Spectra/Por 4 [Spectrum Medical Industries, Houston, TX]) served as a semipermeable barrier between the collagen gel with PMNLs and the glass capillary tube containing the chemoattractant.

Polymorphonuclear leukocyte movement was characterized by analyzing the x and y coordinates of each cell at each time interval. The change in cell position from one time interval to the next was computed as follows: $(\Delta x = x_n - x_{n-1})$ and $(\Delta y = y_n - y_{n-1})$. Both values are in μm. Based on these calculations, the following cell behavioral characteristics were derived: displacement for a time interval $(D = \sqrt{\Delta x^2 + \Delta y^2})$, resultant vector length $(RVL = \sqrt{(x_n - x_i)^2 + (y_n - y_i)^2})$, velocity $(V = D/\text{minute})$, and the angle of displacement with respect to the x-axis $(Dis\ \theta = \tan^{-1}[(\Delta y/\Delta x)])$. The reference angle, derived from the displacement angle, equals 0° when the displacement is in a straight line toward the chemoattractant. Percent motility and stop frequency (percent of motile phase cell was stopped) were also calculated from the data.

To analyze directional movement further, the chemotactic indices was calculated at each minute. The formula for the chemotactic index of motile PMNLs incorporates the resultant vector length times the cosine of the reference angle divided by the total displacement $(\cos\ (Ref\ \theta)\, RVL/\Sigma D)$.

Each measurement was taken from zero to the time interval in question. The relative numbers, derived from this formula, are an index of the straightness of the PMNL path from +1.0 (direct movement toward chemoattractant) to −1.0 (direct movement away from attractant).

Alkali Degradation of Corneas

All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmalic and Vision Research. Bovine eyes were enucleated and frozen within 2 hours of death (Pel-Freez Biologicals, Rogers, AR). These eyes remained frozen until the whole corneas (including epithelium and endothelium) were excised. In a preliminary experiment, three whole corneas were rinsed in HBSS for 30 seconds, their rims were blotted gently on paper towel, their wet weights were determined, and the corneas were dried. The average dry weight of each cornea was calculated to be 83 mg, and the average volume of water in each cornea was 500 μl.

A control experiment was performed to determine if the chemoattractant released from alkali-degraded corneas is endogenous to the uninjured cornea and capable of extraction by rinsing. Thawed corneas were treated with HBSS or alkali for 1 hour because exposure of thawed corneas in buffer for 24 hours at 37°C would pose a real risk of bacterial contamination, cellular disintegration, or both. Two separate groups of six corneas were pooled, rinsed, blotted, and treated with 6 ml of HBSS or 1 N NaOH (final concentration) at 37°C for 1 hour. This yielded

a corneal dry weight to final volume of HBSS or alkali of 1:12. The samples were slowly titrated to pH 7.4 with HBSS or 2.0 N HCl, respectively, while stirring. After centrifugation of each neutralized sample (300*g* for 15 minutes), the supernatants were purified by ultrafiltration and dialysis as described in Ultrafiltration and Dialysis. The final samples, containing substances in the 100 to 1000 molecular weight range, were tested for activity in the polarization assay.

A separate experiment used a 24-hour exposure of corneas to alkali, optimizing the chemoattractant yield. Three whole corneas were pooled, rinsed, and blotted. The corneas were placed in 3 ml of 1.0 N NaOH (final volume and concentration of alkali includes the water content of each cornea) at 37°C for 24 hours. This yielded a corneal dry weight to final volume of 1.0 N NaOH of 1:12. The alkali-degraded corneas were slowly neutralized to pH 7.4 with 2.0 N HCl while stirring. The final volume of the completely dissolved corneas, after acid neutralization, was 4.5 ml. The purification experiment of this crude suspension was performed three times with similar results. A description of the methods used in each case follows.

Ultrafiltration and Dialysis

The crude suspension (1.500 ml) was ultrafiltered through 30,000 MWt cutoff Centriprep (Amicon, Beverly, MA) concentrators at 1200*g* for 90 minutes. This active filtrate (1.250 ml) was then added to Spectra/Por cellulose ester dialysis membranes (Spectrum Medical) with a 100 molecular weight cutoff. The sample was dialyzed twice against 1 liter of distilled water containing 0.05% sodium azide for 1 hour each and once against 1 liter of HBSS for 1 hour. The active isotonic retentate (1.363 ml) was again ultrafiltered through 3000 MWt cutoff Centriprep (Amicon) concentrators at 2500*g* for 1 hour. This active filtrate (1.250 ml) was centrifuged through an Amicon MPS-1 micropartion system with a YM1 Diaflo membrane (1000 MWt). The final active filtrate (1 ml) was further purified by three successive HPLC columns.

High-Performance Liquid Chromatography

The sample (1 ml) was injected into the first Protein PAK I-60 column (Waters Associates, Milford, MA), which separates on the basis of molecular size. The column was eluted by 0.05 M NaCl at 1.0 ml/minute, and the absorbance was monitored at 210 and 280 nm. The eluant was collected into 0.5-ml fractions, and a 0.1-ml aliquot was analyzed for activity in the polarization assay. The remainder of active fraction 24 (0.4 ml) was injected into a μ-Bondapak-C_{18} column (Water Associates, Marlborough, MA) and was eluted by water for 40 minutes at 0.5 ml/minute. Acetonitrile was introduced in the eluant at 40 minutes, and the concentration increased to 100% by 60 minutes. The eluant was monitored at 210 and 280 nm and was collected into 0.5-ml fractions. The peak polarization response was observed in fraction 11. The balance of active fraction 11 (0.4 ml) was injected into a second I-60 column and eluted by 0.05 M NaCl at 0.5 ml/minute. Fractions (0.25 ml) were monitored at 210 and 280 nm.

Amino Acid Analysis

An internal standard (alpha amino butyric acid) was added to an aliquot of the final active fraction. The fraction was dried using vacuum centrifugation and hydrolyzed (in vacuo) using gaseous phase 6 N HCl for 24 hours at 110°C. The sample was dried again and derivitized using phenylisothiocyanate by the standard protocol recommended by the manufacturer for the PicoTag System (Waters Associates). The resultant phenylthiocarbamyl amino acids were analyzed using a Waters Associates PicoTag Column with buffers as recommended by the manufacturer. Standard amino acids (H), 6 N HCl, and phenylisothiocyanate were purchased from Pierce Chemical (Rockford, IL).

Mass Spectrometry

An aliquot of the final active fraction or synthetic peptides was subjected to matrix-assisted laser desorption ionization mass spectral analysis using two different matrices (4-nitrophenol and alpha-cyano-4-hydroxycinnamic acid).

Peptide Sequence Analysis

An aliquot of the final active fraction was applied to a polybrene treated glass fiber filter that had been precycled for four cycles. The sample was then put into the quartz cartridge and into a model 470A Applied Biosystems (Foster City, CA) protein sequencer with an online 120A PTH amino acid analyzer (Applied Biosystems). The methodologies used in the sequencer and the PTH analyzer were as recommended by the manufacturer. All chemicals and supplies used to generate the sequence analysis were purchased from Applied Biosystems.

Treatment of Active Fraction With Prolidase

Prolidase (116,000 MWt) in HBSS was added to the ultrafiltrate or an aliquot of the final active fraction at a concentration of 333 U/150 μl incubation mixture containing 500 μM Ca^{2+} and 600 μM Mg^{2+}. All samples were incubated for 2 or 4 hours at 37°C and maintained at a pH of 8.0. One unit of prolidase will hydrolyze 1.0 μmole of Gly-Pro per minute under these conditions. At the end of the incubation period, each sample was passed through 100,000 MWt cutoff microspin ultrafilter (Lida Manufacturing, Kenosha, WI) to remove the prolidase and the pH of the filtrate returned to 7.4 before testing in the polarization assay.

Synthetic Peptides

Nine possible permutations of free, acetylated, or methylated N-terminal tripeptides with two prolines (P) and one glycine (G) were synthesized. The peptides (N-acetyl-PGP, N-acetyl-PPG, N-acetyl-GPP, PGP, PPG, GPP, N-methyl-PGP, N-methyl-PPG, and N-methyl-GPP) were manually synthesized on a Boc-Pro or Boc-Gly Merrifield resin (Millipore, Bedford, MA). Ninhydrin tests were used after each deblocking and coupling step to check for desired results. Amino acids were coupled using routinely available t-Boc synthetic techniques. N-methyl-amino acids were purchased from Sigma, but acetylated peptides were capped after removal of the t-Boc group.

The finished trimer was dried under vacuum and cleaved with anhydrous hydrogen fluoride. The cleaved product was washed with ether and extracted with 40% acetic acid. Both the ether wash and the acid wash were freeze dried. Mass spectral analysis indicated that the masses found in both washes agreed precisely with the expected masses of the target products.

Peptide samples were dissolved in 3% acetic acid and dialyzed (1 ml/l) in Spectra/Por cellulose ester membranes with a 100-MWt cutoff (Spectrum) against distilled water with 0.05% sodium azide for 6 hours (changed every hour) at room temperature, distilled water with 0.05% sodium azide for 16 hours at 0°C to 4°C, and HBSS for 4 hours (changed every hour) at room temperature.

RESULTS

Purification of the Chemoattractant(s) From Alkali-Degraded Corneas

The corneal samples treated with HBSS for 1 hour showed no significant activity (polarization index, mean ± SEM, $n = 5$: 10 μl = 1.2 ± 0.8, 25 μl = 1.8 ± 1.9, 50 μl = 1.3 ± 1.0, and 100 μl = 2.9 ± 2.4), whereas the corneas treated with alkali for 1 hour demonstrated a high degree of activity (polarization index, mean ± SEM, $n = 5$: 10 μl = 14.9 ± 5.1, 25 μl = 54.7 ± 7.2, 50 μl = 87.8 ± 8.0, and 100 μl = 98.3 ± 8.1).

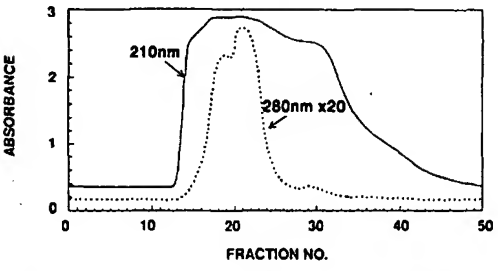


FIGURE 2. Chromatography of the polymorphonuclear leukocytechemoattractant from alkali-degraded corneas on Protein PAK I-60 high-performance liquid chromatography column. One milliliter of the final active ultrafiltrate, containing molecules in the 100 to 1000 range, was injected into this first high-performance liquid chromatography column. The column was eluted by 50 mM NaCl at 0.5 ml/minute. Fractions (0.5 ml) were collected and the absorbance monitored at 210 and 280 nm. The absorbance at 280 nm was magnified 20 times. The polarization assay detected peak polymorphonuclear leukocyte polarization in fraction 24 (0.5 ml).

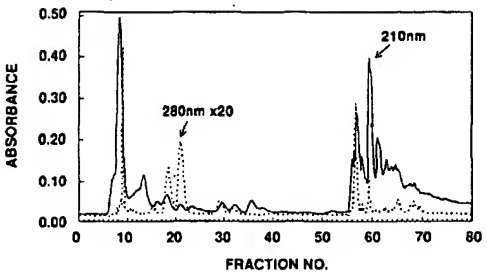


FIGURE 3. Chromatography of the polymorphonuclear leukocyte chemoattractant from alkali-degraded corneas on μ-Bondapak-C_{18} high-performance liquid chromatography column. The remainder of active fraction 24 (0.4 ml) from the first column was injected into a μ-Bondapak-C_{18} column and eluted in high-performance liquid chromatography grade water. Fractions (0.5 ml) were collected at 0.5 ml/minute, and the absorbance was detected at 210 and 280 nm. The absorbance at 280 nm was magnified 20 times. Acetonitrile was introduced in the eluant at fraction 40, and the concentration increased to 100% by fraction 60. The peak polarization response was demonstrated in fraction 11 (0.5 ml).

The crude suspension from corneas degraded by alkali for 24 hours was ultrafiltered and dialyzed to produce an isotonic sample containing molecules in the 100- to 1000-MWt range. This low molecular weight ultrafiltrate retained 84% of the polarization activity found in the original ultrafiltrate (<30,000 MWt). Time–response curves for three doses of the low molecular weight ultrafiltrate demonstrated that the peak polarization response occurred at 3 to 5 minutes and decreased at 15 and 45 minutes (Fig. 1). Only 16% of the original polarization activity was detected in the retentates containing molecules in the 1000- to 30,000-MWt range. Retentates with >30,000 MWt were not analyzed for polarization or chemotactic activity because of the presence of a high molecular weight stimulant of the PMNL respiratory burst,[17] which overrides the chemotactic response.

After injection of the 100- to 1000-MWt ultrafiltrate into the first I-60 HPLC column, the PMNL polarization assay detected peak activity in fraction 24 and moderate activity in fraction 25 (Fig. 2). The remainder of fraction 24 (0.4 ml) was injected into a μ-Bondapak-C_{18} column. Activity was demonstrated by the polarization assay in fractions 10 to 12, with the peak at fraction 11 (Fig. 3). The balance of fraction

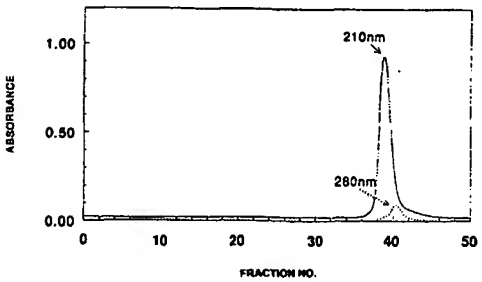


FIGURE 4. Chromatography of the polymorphonuclear leukocyte chemoattractant from alkali-degraded corneas on Protein PAK I-60 high-performance liquid chromatography column. The balance of active fraction 11 (0.4 ml) from the C_{18} column was injected into a second I-60 column and eluted by 0.05 M NaCl. Fractions (0.25 ml) were collected at 0.5 ml/minute. The absorbance of each fraction was monitored at 210 and 280 nm. The polarization assay detected an equal amount of activity in fractions 38 and 39 (0.25 ml each).

11 (0.4 ml) was rechromatographed on the Protein PAK I-60 column. The chromatogram demonstrated one major 210-nm absorption peak occurring in fractions 38 and 39, and one minuscule 280-nm peak appearing in fraction 39 (Fig. 4). The polarization assay detected an equal amount of activity in fractions 38 and 39. Fraction 38 is likely to be more pure because it has little absorbance at 280 nm. The molecular weight of active fraction 38 is located between the standards tyrosine (181) and reduced glutathione (307), suggesting a size in this general range (Fig. 5). Comparison of the specific activities calculated for the

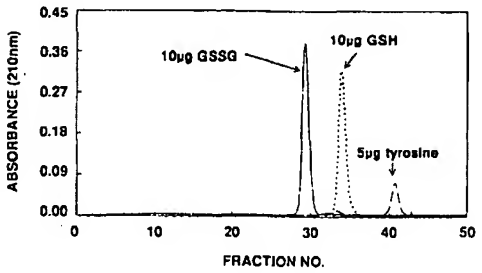


FIGURE 5. Chromatography of standards on Protein PAK I-60 high-performance liquid chromatography column. The column was eluted by 0.05 M NaCl and 0.25 ml fractions were collected at 0.5 ml/minute. The absorbance was monitored at 210 nm. The location of the molecular weight standards, tyrosine (181) and reduced glutathione (307), suggest that active fraction 38 is within this molecular weight range. GSH = reduced glutathione; GSSG = oxidized glutathione.

TABLE 2. Amino Acid Analysis of Fraction 38

Amino Acid	*Percentage*
Proline	52.3
Glycine	35.1
Alanine	5.1
Hydroxyproline	3.9
Serine	1.6
Leucine	1.1
Arginine	1.0

crude ultrafiltrate (<30,000 MWt cutoff, and the purified active fraction (#38) showed a 6875-fold purification of the chemoattractant. The remainder of fraction 38 was used to analyze the molecular characteristics and chemotactic activity of the chemoattractant.

The ultrafiltrate (100 to 3000 MWt cutoff) or active fraction 38 was mixed with prolidase (E.C. 3.4.13.9) and incubated for 2 or 4 hours. After the 4-hour incubation, PMNL polarization activity was decreased by 88% for the ultrafiltrate sample and 77% for fraction 38 (Table 1). This highly specific enzymatic reaction, cleaving Pro-X (X = amino acid) peptide bonds, is strong evidence that the chemoattractant is a peptide containing the Pro-X peptide bond.

Analysis of fraction 38 shows approximately twice as much proline as glycine based on amino acid composition by the Picotag method. There are no other amino acids present in high enough percentages to be part of this small peptide (Table 2). The above information strongly suggested that the chemoattractant is a tripeptide with two prolines and one glycine.

N-terminal sequence analysis was unable to establish a primary sequence, but Picotag analysis showed an abundance of sample. This indicates blockage of the N-terminal amino acid residue.

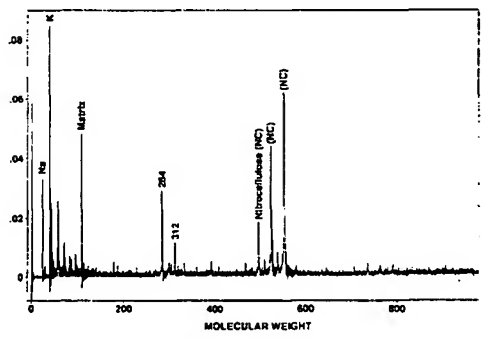


FIGURE 6. Matrix-assisted laser desorption ionization mass spectral analysis of active fraction 38, using a 4-nitrophenol matrix. The two unknown molecules are at molecular weights 312 and 284.

TABLE 3. Specific Activity of Synthetic Peptides in Polymorphonuclear Leukocyte Polarization Assay

Synthetic Peptide	*Specific Activity*
n-acetyl-PGP	17.0
n-methyl-PGP	9.7
PGP	4.4
PPG	2.7
n-acetyl-GPP	1.7
GPP	0.7
n-methyl-GPP	0.2
n-methyl-PPG	0.2
n-acetyl-PPG	Not detectable
Active fraction 38	21.0

One unit activity was defined as the amount of sample required to generate a 50% polarization index. Specific polarization activity was calculated as units of activity per milligram peptide.

Matrix-assisted laser desorption ionization mass spectrometry showed that fraction 38 contains only two unknown molecules of 312 MWt and 284 MWt (Fig. 6). Two different, but mutually confirmatory, matrices were used.

Synthetic Peptides

The polarization response of PMNLs was used to screen synthetic peptide samples before selected chemoattractant studies in the CG–VCA system. In summary, Table 3 shows that N-acetyl-PGP and N-methyl-PGP produced a powerful response in the polarization assay approximately an order of magnitude greater than the less active peptides. The specific activity of N-acetyl-PGP was similar to the purified chemoattractant in fraction 38. One unit activity was defined as the amount of sample required to generate a 50% polarization index. Specific polarization activity was calculated as units of activity per milligram of peptide. Note that the molecular weights of n-acetyl-PGP and n-methyl-PGP are 312 and 284 respectively, the same as that noted by mass spectral analysis of the purified chemoattractant.

Time–response curves for three doses of N-acetyl-PGP and N-methyl-PGP demonstrated that the peak polarization response was at 3 to 5 minutes for each dose of both tripeptides, decreasing at 15 and 45 minutes (Figs. 7, 8).

Chemotactic Activity of Fraction 38 and Synthetic Peptides

Polymorphonuclear leukocyte chemotaxis was demonstrated in the CG–VCA system using the purified active fraction (38) from alkali-degraded corneas (Fig. 9). The chemotactic index reached a peak at 2 minutes, decreasing for the remaining 8 minutes of the experiment.

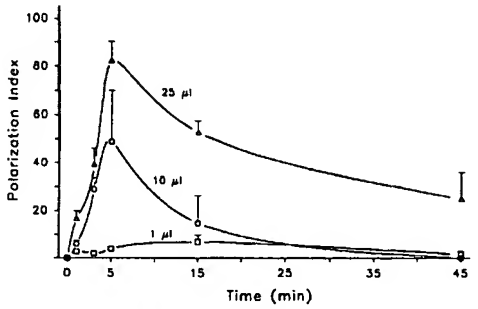


FIGURE 7. Time–response curves for the polarization index of polymorphonuclear leukocytes exposed to three doses of N-acetyl-PGP. The negative control value (polymorphonuclear leukocytes in buffer only) was subtracted from the total polarization score to achieve a polarization index for each sample. The final concentrations of N-acetyl-PGP in the 250-µl incubation mixtures were 1.1×10^{-5} M (1 µl), 1.1×10^{-4} M (10 µl), and 2.8×10^{-4} M (25 µl). Each data point represents the mean ± standard error of the mean, $n = 5$. Volumes of n-acetyl PGP in excess of 25 µl produce polymorphonuclear leukocyte lysis, possibly as a result of residual reagents from the peptide synthesis technique.

N-acetyl-PGP demonstrated PMNL chemotaxis in the CG–VCA system (Fig. 10), comparable to the response found with fraction 38. A significantly positive chemotaxis was noted from 7 to 10 minutes. There was a delay in the chemotactic response, probably rep-

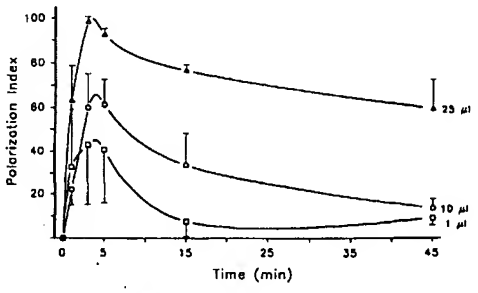


FIGURE 8. Time–response curves for the polarization index of polymorphonuclear leukocytes exposed to three doses of N-methyl-PGP. The negative control value (polymorphonuclear leukocytes in buffer only) was subtracted from the total polarization score to achieve a polarization index for each sample. The final concentrations of N-methyl-PGP in the 250 µl incubation mixtures were 6.0×10^{-5} M (1 µl), 6.0×10^{-4} M (10 µl), and 1.5×10^{-3} M (25 µl). Each data point represents the mean ± standard error of the mean, $n = 5$. Volumes of N-methyl PGP in excess of 25 µl produce polymorphonuclear leukocyte lysis, possibly as a result of residual reagents from the peptide synthesis technique.

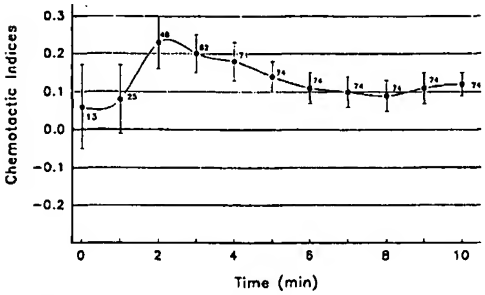


FIGURE 9. Chemotactic index of polymorphonuclear leukocytes exposed to purified active fraction 38 from alkali-degraded corneas. Active fraction (1.8×10^{-4} M, total estimated concentration of N-methyl-PGP and N-acetyl-PGP from Picotag analysis of amino acid composition) was added to the glass capillary tube. Each data point represents the chemotactic indices (mean ± SEM) for both 30-second intervals at each minute for all motile polymorphonuclear leukocytes from three separate experiments. This number of motile cells is included adjacent to the data point for each time period. Ninety-nine cells were tracked. The single sample Student's *t*-test was used to determine if the chemotactic index for each minute differed significantly from zero (greater than zero from 2 to 10 minutes, $P < 0.001$). Polymorphonuclear leukocytes also were tracked for 5 minutes before the addition of the chemoattractant as a control, but the number of motile cells was low, and their movement was not significantly different from zero (random).

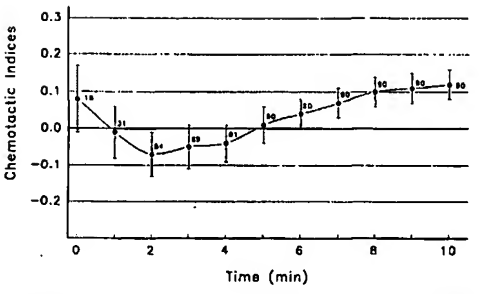


FIGURE 10. Chemotactic index of polymorphonuclear leukocytes exposed to synthetic N-acetyl-PGP. N-acetyl-PGP (2.8×10^{-3} M) was added to the glass capillary tube. Each data point represents the chemotactic indices (mean ± SEM) for both 30-second intervals at each minute for all motile polymorphonuclear leukocytes from three separate experiments. This number of motile cells is included adjacent to the data point for each time period. One hundred three cells were tracked. The single sample Student's *t*-test was used to determine if the chemotactic index for each minute differed significantly from zero (greater than zero at 7 [$0.01 < P < 0.02$], 8, 9, and 10 minutes [$P < 0.001$]). Polymorphonuclear leukocytes also were tracked for 5 minutes before the addition of the chemoattractant as a control, but the number of motile cells was low, and their movement was not significantly different from zero (random).

resenting a nonoptimal gradient of the chemoattractant in the chemotactic chamber.

A similar chemotactic response was recorded for PMNLs exposed to N-methyl-PGP in the CG–VCA system (Fig. 11). A moderate chemotaxis was reached at 3 minutes and maintained thereafter.

The behavioral characteristics of PMNLs exposed to the active fraction 38, N-acetyl-PGP, and N-methyl-PGP are summarized in Table 4. There was a dramatic increase in the percent motility of each PMNL population after the addition of each chemoattractant. The stop frequency of PMNLs during the 5- to 10-minute period after the addition of each chemoattractant showed a significant decrease ($P < 0.001$, Student's *t* test) when compared to the 5-minute control period. Based on the Student's *t*-test, the velocity of PMNL movement during the 5- to 10-minute period was significantly increased over the 5-minute control period for N-acetyl-PGP ($0.02 < P < 0.05$), N-methyl-PGP ($P < 0.001$), and active fraction 38 ($P < 0.001$). When compared to the respective 5-minute control periods, PMNL displacement was not significantly increased during the 5- to 10-minute period for N-acetyl-PGP, but it was highly significant ($P < 0.001$) for N-methyl-PGP and the active fraction.

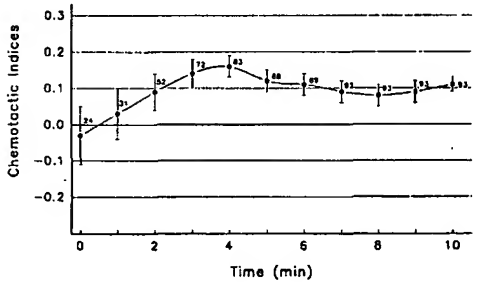


FIGURE 11. Chemotactic index of polymorphonuclear leukocytes exposed to synthetic N-methyl-PGP. N-methyl-PGP (1.5×10^{-2} M) was added to the glass capillary tube. Each data point represents the chemotactic indices (mean ± SEM) for both 30-second intervals at each minute for all motile polymorphonuclear leukocytes from three separate experiments. This number of motile cells is included adjacent to the data point for each time period. One hundred five cells were tracked. The single sample Student's *t*-test was used to determine if the chemotactic index for each minute differed significantly from zero (greater than zero from 3 to 10 minutes [$P < 0.001$]). Polymorphonuclear leukocytes also were tracked for 5 minutes before the addition of the chemoattractant as a control, but the number of motile cells was low, and their movement was not significantly different from zero (random).

TABLE 4. Comparison of Behavioral Characteristics

	Motility (%)	*Stop Frequency (%)*	*Velocity (μm/min)*	*Displacement (μm)*
Control				
(−5 to 0 min)	17.5	34.5 ± 5.7	7.4 ± 0.4	28.1 ± 3.7
n-acetyl-PGP				
(0 to 5 min)	87.4	24.6 ± 1.9	7.1 ± 0.2	27.3 ± 1.5
(5 to 10 min)	87.4	17.1 ± 1.9	8.5 ± 0.2	36.0 ± 1.7
Control				
(−5 to 0 min)	21.9	41.8 ± 5.2	6.6 ± 0.4	25.9 ± 2.6
n-methyl-PGP				
(0 to 5 min)	87.4	17.4 ± 1.9	9.2 ± 0.2	33.7 ± 1.6
(5 to 10 min)	88.6	13.2 ± 1.5	9.9 ± 0.2	44.0 ± 1.6
Control				
(−5 to 0 min)	16.2	28.3 ± 7.2	5.4 ± 0.4	14.7 ± 2.8
Active Fraction				
(0 to 5 min)	74.7	24.3 ± 2.0	6.8 ± 0.2	26.6 ± 1.6
(5 to 10 min)	74.7	12.5 ± 1.5	8.7 ± 0.2	41.4 ± 1.5

Total number of tracked polymorphonuclear leukocytes for experiments activated with n-acetyl PGP (103), n-methyl PGP (105), and the active fraction (99).

DISCUSSION

Our previous studies[9,10,11] demonstrated the presence of PMNL chemoattractant(s) generated from alkali-degraded whole corneas. Its small size and hydrophilic nature imparted ideal characteristics for the rapid diffusion of the molecule through collagenous tissues.[11] We conclude from the current study that the PMNL chemoattractants, isolated from these alkali-degraded corneas, are acetylated and methylated tripeptides (Pro-Gly-Pro). The absence of activity in corneas rinsed with HBSS demonstrated that the chemoattractants were not endogenous to the uninjured cornea.

The chemoattractant(s) released from alkali-degraded corneas, as well as the synthetic N-acetyl-Pro-Gly-Pro and the N-methyl-Pro-Gly-Pro, induced a peak polarization response by PMNLs within 5 minutes that decreased dramatically after 15 and 45 minutes. The basis for declining polarization activity at the latter time periods is unknown. It might indicate that the cell membrane receptors of the PMNL were saturated with the chemoattractant or that the cell was metabolizing the chemoattractant.

Alkali degrades the cornea into numerous heterogeneous peptides separable by column chromatography. Passage of the alkali-degraded corneal ultrafiltrate through a protein PAK I-60 column and then the μ-Bondapak-C_{18} column removed a large amount of the inactive peptides. A highly purified fraction (38) with chemotactic activity for PMNLs was recovered upon rechromatography on the protein PAK I-60 column.

The PMNL chemoattractant(s) was determined to be a peptide with at least one Pro-X peptide bond, based on the loss of biologic activity after incubation with prolidase. The amino acid composition studies showed that the sample contained twice the amount of proline than glycine. Mass spectrometry revealed that only two unknown molecular species were present, with masses at 312 and 284, respectively. This information strongly suggested that the chemoattractant was a tripeptide with two prolines and one glycine. There were no other amino acids present in high enough percentages to be part of this peptide. An attempt at N-terminal sequencing failed, establishing that both tripeptides are probably N-terminal blocked. Calculation of the molecular weight of two prolines and one glycine, and acetylation or methylation of the N-terminal group, agrees exactly with the molecular weights of 312 and 284 found by mass spectrometry.

The limited permutations of free N-terminal, N-acetylated, or N-methylated tripeptides, containing two prolines and one glycine, made it practical to synthesize all nine possible sequences. Polarization assay of PMNLs exposed to these samples showed that only N-acetyl-PGP and, to a lesser degree, N-methyl-PGP yielded a potency comparable to the purified chemoattractant. Further analysis of both synthetic tripeptides in the CG–VCA system revealed chemotactic activity comparable to that of the purified chemoattractant. These results confirm that N-acetyl-PGP and N-methyl-PGP are PMNL chemoattractants. These data show that the alkali-generated chemoattractant and the synthetic chemoattractants are the same.

Acetylation[18,19] is the most common form of naturally occurring N-terminal peptide blockage, but methylation[20] also occurs. Any of these acetylated or methylated proteins with the sequence Pro-Gly-Pro from the amino terminus could be a source of the

chemoattractant. Alternately, exposure to alkali is known to cleave the ester-linked acetyl group from naturally occurring neutral peptides, potentially making these groups available to alkali-generated peptides with a free N-terminus.[19] Alkali has been shown to cleave protein (including collagen) into heterogeneous peptide units.[21] The amino terminus of these residual peptides would be susceptible to acetylation or methylation under these alkaline conditions. In fact, peptides are routinely acetylated at the N-terminus under alkaline conditions during peptide synthesis.[22,23] The results of experiments in the current study show that this process could occur in the alkali-degraded cornea. Therefore, a parallel can be drawn for this process in the alkali-injured cornea.

Identification of the primary sequence of the purified chemoattractant opens an important door to basic studies and understanding of PMNL influx into the alkali-injured cornea. This knowledge will allow the synthesis of relatively large amounts of the chemoattractant for extensive testing. It also may allow for a rational plan of attack to defeat the destructive PMNL infiltration into the alkali-injured cornea by developing inhibitors of the chemoattractant. In this regard, the synthetic N-acetyl-Pro-Pro-Gly has no apparent chemotactic activity and, hence, is a candidate as a chemoattractant inhibitor. The other synthetic tripeptides with specific chemotactic activity <1.0, N-methyl-PPG, N-methyl-GPP, and unblocked GPP, might also be potential inhibitors. The precedent for this approach was set in an experiment in which PMNL accumulation, induced by injection of N-formyl-methionyl-leucyl-phenylalanine into normal cornea, was inhibited by the simultaneous injection of inactive, synthetically produced analogues.[24] The search for inhibitors might be focused by using structure-based drug design,[25] a recent innovative approach for developing new drugs as therapeutic agents.

The tripeptide P-G-P probably originates from a protein that is degraded by alkali. The National Center for Biotechnology Information Peptide Sequence Data Base was consulted to determine the number of proteins that contain the P-G-P sequence and its frequency in particular proteins.[26] Numerous mammalian proteins contain this sequence, including collagen, proteoglycans, fibronectin, laminin, ICAM-1, integrin, and Na^+K^+ ATPase. Collagen is one of the likeliest sources because of the relative frequency of this amino acid sequence in collagen and the fact that a relatively high proportion of corneal protein is collagen. The triple helical portion of the collagen molecule contains glycine in every third residue, with proline and hydroxyproline appearing next most frequently in the sequence. When the amino acid residues are cleaved, hydrolysis of the peptide bond renders a free carboxyl terminal while simultaneously, or shortly thereafter, methylation or acetylation of the n-terminal group might occur. The Pro-Gly-Pro sequence does exist in this portion of the collagen molecule.

Our current findings that N-acetyl-PGP and N-methyl-PGP induce chemotaxis in resting PMNL are the direct result of experiments designed to identify the chemotactic activity in alkali-degraded whole corneas. Earlier studies showed a locomotory response by fibroblasts to collagen-derived peptides, especially tripeptide sequences selected for study based on their common appearance in the collagen molecule.[27] Using similar reasoning, Laskin et al[28] also synthesized tripeptides, including combinations of Pro, Hyp and Gly, which activated PMNL locomotion in selected sequences. Polytripeptides, composed of 5 or 10 recurring units of Pro-Pro-Gly and Pro-Hyp-Gly, were the most active. Blocking of the amino or carboxyl end of the molecules changed the activity. No PGP sequence was tested. It is now known that the methodology used in the former experiment could only show locomotion of fibroblasts, whereas the latter experiment used checkerboard analysis of the Boyden chamber assay, a questionable method for simulating PMNL chemotaxis in vivo. The true chemotactic properties of these compounds, as measured by more recent and accurate visual techniques, might be regarded as unknown. In spite of these considerations, this family of compounds might indeed induce chemotaxis by attaching to the same PMNL membrane receptor as N-acetyl-PGP and N-methyl-PGP.

It is likely that the alkali-generated tripeptide chemoattractant(s) discussed in this article play a significant role in triggering the early neutrophil response after an alkali injury. Other low molecular weight chemoattractants, such as platelet-activating factor[29] and LTB_4,[30] are known to be present in the experimental alkali-injured eye and may be active in the further recruitment and accumulation of neutrophils. It is yet to be determined whether other corneal inflammatory diseases generate the same tripeptide chemoattractant(s) as those discovered in the current study. These tripeptide chemoattractant(s) might be the common denominator in inflammatory conditions when degradation of collagen or other PGP-containing proteins is involved. Discovery of the alkali-generated chemoattractant(s) might lead to a fuller understanding of chemotaxis in alkali-injuries as well as in other eye diseases and inflammation in other tissues of the body.

Key Words

chemotaxis, chemotactic peptides, synthetic peptides, polymorphonuclear leukocytes, alkali injury, cornea, ulceration

Acknowledgments

We gratefully acknowledge the expert technical assistance of the Biotechnology Support Laboratory at the Texas Agriculture Experiment Station (Texas A & M University, College Station, Texas) in the areas of amino acid analysis, mass spectrometry, peptide sequence analysis, and peptide synthesis. We especially wish to thank Dr. Mark S. Wright for his many consultations on matters of peptide analysis.

References

1. Hughes WF Jr. Alkali burns of the eye: I: Review of the literature and summary of present knowledge. *Arch Ophthalmol.* 1946;35:423–449.
2. Brown SI, Weller CA. The pathogenesis and treatment of collagen-induced diseases of the cornea. *Trans Am Acad Ophthalmol Otolaryngol.* 1970;74:375–383.
3. Kenyon KR, Berman M, Rose J, Gage J. Prevention of stromal ulceration in the alkali-burned rabbit cornea by glued-on contact lens: Evidence for the role of polymorphonuclear leukocytes in collagen degradation. *Invest Ophthalmol Vis Sci.* 1979;18:570–587.
4. Foster CS, Zelt RP, Mai-Phan T, Kenyon KR. Immunosuppression and selective inflammatory cell depletion: Studies of a guinea pig model of corneal ulceration after ocular alkali burning. *Arch Ophthalmol.* 1982;100:1820–1824.
5. Pfister RR, Burstein N. The alkali burned cornea: I: Epithelial and stromal repair. *Exp Eye Res.* 1976; 23:519–535.
6. Pfister RR, Nicolaro ML, Paterson CA. Sodium citrate reduces the incidence of corneal ulcerations and perforations in extreme alkali-burned eyes-acetylcystine and ascorbate have no favorable effect. *Invest Ophthalmol Vis Sci.* 1981;21:486–490.
7. Pfister RR, Haddox JL, Paterson CA. The efficacy of sodium citrate in the treatment of severe alkali burns of the eye is influenced by the route of administration. *Cornea.* 1982;1:205–211.
8. Paterson CA, Williams RN, Parker AV. Characteristics of polymorphonuclear leukocyte infiltration into the alkali burned eye and the influence of sodium citrate. *Exp Eye Res.* 1984;39:701–708.
9. Pfister RR, Haddox JL, Dodson RW, Harkins LE. Alkali burned collagen produces a factor stimulatory to PMN. ARVO Abstracts. *Invest Ophthalmol Vis Sci.* 1984;25:6.
10. Pfister RR, Haddox JL, Dodson RW, Harkins LE. Alkali-burned collagen produces a locomotory and metabolic stimulant to neutrophils. *Invest Ophthalmol Vis Sci.* 1987;28:295–304.
11. Pfister RR, Haddox JL, Sommers CI. Alkali-treatment of cornea generates a low molecular weight chemoattractant for polymorphonuclear leukocytes. *Invest Ophthalmol Vis Sci.* 1993;34:2297–2304.
12. Ferrante A, Thong YH. A rapid one-step procedure for purification of mononuclear and polymorphonuclear leukocytes from human blood using a modification of the Hypaque–Ficoll technique. *J Immunol Methods.* 1978;24:389–397.
13. Pfister RR, Haddox JL, Harkins LE, Dodson RW. The effects of citrate on fMLP induced polymorphonuclear leukocyte stimulation and locomotion. *Cornea.* 1984/1985;3:183–188.
14. Haston WS, Shields JM. Neutrophil leukocyte chemotaxis: A simplified assay for measuring polarizing responses to chemotactic factors. *J Immunol Methods.* 1985;81:229–237.
15. Haddox JL, Pfister RR, Sommers CI. A visual assay for quantitating neutrophil chemotaxis in a collagen gel matrix: A novel chemotactic chamber. *J Immunol Methods.* 1991;141:41–52.
16. Haddox JL, Knowles IW, Sommers CI, Pfister RR. Characterization of chemical gradients in the collagen gel-visual chemotactic assay. *J Immunol Methods.* 1994;171:1–14.
17. Pfister RR, Haddox JL, Barr DY, Berry S, and Lam K-W. Amino acid composition of a neutrophil respiratory burst stimulant: Evidence for a protein, noncollagenous source. *Invest Ophthalmol Vis Sci.* 1991;32:2112–2118.
18. Brown JL, Roberts WK. Evidence that approximately eighty per cent of the soluble proteins from Ehrlich ascites cells are Nalpha-acetylated. *J Biol Chem.* 1976;251:1009–1014.
19. Endo Y. In vivo deacetylation of N-acetyl amino acids by kidney acylases in mice and rats: A possible role of acylase system in mammalian kidneys. *Biochim Biophys Acta.* 1980;628:13–18.
20. Stryer L. Biosynthesis of amino acids and Heme. In: *Biochemistry.* New York: WH Freeman; 1988:582.
21. Berry SM, Hong BS, Lam K-W, Haddox JL. Pfister RR. Degradation of bovine corneal collagen by alkali. *Cornea.* 1989;8:150–154.
22. Bodanszky M. Protection of functional groups. In: Peptide Chemistry: A practical textbook. New York: Springer–Verlag; 1988:80.
23. Riordan JF, Vallee BJ. Acetylation. In: *Methods in Enzymology.* 1967;11:565–570.
24. Ben-Zvi A, Rodrigues M, Gery I, Schiffmann E. Induction of inflammation by synthetic mediators. In: Suran A, Gery I, Nussenblatt RB (eds): *Immunology of the Eye: Workshop: III.* Arlington, VA: Information Retrieval; 1981:245.
25. Bugg CE, Carson WM, Montgomery JA. Drugs by design. *Sci Am.* 1993;269:92–98.
26. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol.* 1990;215:403–410.
27. Postlethwaite AE, Seyer JM, Kang AH. Chemotactic attraction of human fibroblasts to type I, II, and III collagens and collagen-derived peptides. *Proc Natl Acad Sci USA.* 1978;75:871–875.
28. Laskin DL, Kimura T, Sakakibara S, Riley DJ, Berg RA. Chemotactic activity of collagen-like polypeptides for human peripheral blood neutrophils. *J Leukocyte Biol.* 1986;39:255–266.
29. Bazan HEP, Reddy STK, Lin N. Platelet-activating factor (PAF) accumulation correlates with injury in the cornea. *Exp Eye Res.* 1991;52:481–491.
30. Borodic G, Conte J, Aswad M, et al. Aqueous humor leukotriene levels in animal models of inflammation. ARVO Abstracts. *Invest Ophthalmol Vis Sci.* 1985;26:99.

Institution (BRAIN).

Top

Nature

ISSN: 0028-0836

EISSN: 1476-4687

- **About NPG**
- **Contact NPG**
- **Nature jobs.com**
- **Privacy policy**
- **Legal notice**
- **Accessibility statement**
- **RSS web feeds**
- **Help**

the prior art. It is immaterial as to the method for producing the products as long as the products meet the claimed tripeptides absent a showing to the contrary. The arguments have been deemed not to be persuasive to withdraw the rejections which stand as recited:

> "1. Ito et al "A tripeptides 'anticodon' deciphers stop codons in messenger RNA", Nature 403 pg 680-684 (February 10, 2000);
> 2. Pfister, E.A., "Identification and Synthesis of Chemotactic Tripeptides From Alkali-Degraded Whole Cornea A Study of N-Acetyl-Proline-Glycine-Proline and N-Methyl-Proline-Glycine-Proline," Investigative Ophthalmology and Visual Science; Jun. 1995; 36(7): 1306-16 ;
> 3. Haddox et al., U.S. 6,310,041 ;
> 4. St. Pierre et al., U.S. 5,856,308.
>
> Each of the references teaches a product having 100% mol of isolated tripeptides containing a terminal proline that is considered to be within the scope of all of the claimed products.
>
> **St. Pierre et al** discloses the following:
>
> In attempts to clarify the correlation between the primary and secondary structures of collagen, a variety of polypeptides with repeating sequences (Pro-Pro-Gly), (Pro-Hyp-Gly), and others have been synthesized and evaluated during the past twenty years.
>
> **Haddix et al** teaches the following:
>
> > Preferably, the neutrophil chemoattractant is selected from the group consisting of N-acetyl-PGP, N-acetyl-PGX, N-methyl-PGX, N-methyl-PGP and small peptide chemoattractants containing proline and glycine.
> >
> > Five complementary peptides were tested as potential inhibitors of **N-acetyl-PGP**: ------ RTR (SEQ ID NO:2), RTRGG (SEQ ID NO:3), RTR dimer, RTR tetramer, and ASA (SEQ ID NO:4) tetramer. In addition, the RTR tetramer and both monomeric peptides (RTR and RTRGG) were tested, separately, for inhibition of the ultrafiltered tripeptide chemoattractants or LTB.sub.4 ----- .;